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"PMN-Elastase Assay": Enzyme Immunoassay for Human Polymorphonuclear Elastase Complexed with α_1-Proteinase Inhibitor

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Summary: A solid phase, enzyme-linked immunoassay is described for the quantitative determination of the complex of human granulocyte elastase (EC 3.4.21.37) with α_1-proteinase inhibitor. The assay employs antibody-coated test tubes and it is suitable for routine use in clinical chemistry laboratories. Data for sample stability and test characteristics are given. A reference range of 20–180 µg/l elastase in plasma was determined. The diagnostic significance of granulocyte elastase levels in plasma in inflammatory diseases is discussed.

„PMN-Elastase Assay": Enzymimmunassay zur Bestimmung des Komplexes von menschlicher Granulocyten-Elastase mit α_1-Proteinaseinhibitor

Zusammenfassung: Es wird ein Festphasen-Enzymimmunoassay zur quantitativen Bestimmung des Komplexes aus menschlicher Granulocyten-Elastase und α_1-Proteinaseinhibitor beschrieben. Es handelt sich um eine routinefähige Testversion mit Antikörper-beschichteten Röhrchen. Daten zur Stabilität von Proben und die Testcharakteristica werden angegeben. Als Referenzbereich für Elastase im Plasma wurden 20–180 µg/l ermittelt. Die Bedeutung der Granulocyten-Elastase im Plasma für die Diagnose und Verlaufskontrolle von entzündlichen Prozessen wird diskutiert.

Introduction

Human polymorphonuclear neutrophilic leukocytes contain large amounts of neutral proteinases which are released when the granulocytes are exposed to a phagocytic stimulus. In inflammatory diseases local imbalance between these proteinases and available proteinase inhibitors may cause tissue injury as well as degradation of plasma proteins (1). Granulocyte elastase (EC 3.4.21.37) is of special pathological interest because of its high concentration and its broad specificity for a variety of connective tissue components (i.e. elastin, collagen, proteoglycans) and plasma proteins (i.e. IgG, complement factors C_3 and C_5, various clotting factors), cf. l.c. (2, 3). Clearly, the quantitative determination of elastase in inflamed tissue and in the circulating blood should provide information on connective tissue diseases, but only a few reports have so far been published on quantitative assays for the detection and quantitation of elastase in biological fluids (4–7, 18). The main inactivating agent in plasma for elastase is α_1-proteinase inhibitor (8). Elastase interacts with the inhibitor to form a complex with a molar ratio of 1 to 1 and a molecular weight of approximately 80.000 Daltons (8).

We developed a solid phase enzyme-linked immunoassay for the determination of the complex of elastase with α_1-proteinase inhibitor (9, 10). In this paper we describe a rapid assay version in test tubes for routine use in the clinical chemistry laboratory.

Materials and Methods

Reagents

All reagents were from E. Merck, unless stated otherwise.

Sepharose 6 B® and Concanavalin A-Sepharose® were from Deutsche Pharmacia; CM-Cellulose CM 52 was from Whatman Biochemicals Ltd; Methoxysuccinyl-*L*-ala-*L*-ala-*L*-pro-*L*-val-4-nitroanilide was from Bachem AG.

Granulocyte elastase and antibodies

Elastase was isolated from granule extracts of normal human granulocytes by a procedure described previously (11). The preparation was free of contaminant granular or serum proteins as shown by polyacrylamide gel electrophoresis at pH 4.5, and by immunoelectrophoresis with antisera against granular extract and human serum.

Antisera against elastase were raised in sheep and were shown to be antigen-specific by immunoelectrophoresis. The immunoglobulin G fraction was isolated with the use of caprylic acid and ion-exchange fractionation.

α_1-Proteinase inhibitor and antibodies

Human α_1-proteinase inhibitor was isolated from outdated plasma. The purification procedure consisted of affinity chromatography on Fractogel® TSK-Red, ion exchange chromatography on Fractogel® TSK DEAE-650, and gel filtration on Fractogel® TSK HW-55 according to l.c. (12). The resulting preparation of α_1-proteinase inhibitor was further purified by chromatography on Concanavalin A-Sepharose® (2.6 cm × 20 cm), previously equilibrated with 50 mmol/l Tris/HCl buffer pH 7.5 containing 1 mmol/l $MgCl_2$, 1 mmol $MnCl_2$, and 1 mmol/l $CaCl_2$. Unbound material was washed from the column with the same buffer. α_1-Proteinase inhibitor was eluted by adding 0.1 mol/l methyl α-*D*-mannoside to the buffer. The final protein preparation was homogeneous as judged by a polyacrylamide gel electrophoresis at pH 9.0, and by immunoelectrophoresis. Antisera against α_1-proteinase inhibitor were raised in rabbits and were shown to be antigen-specific by immunoelectrophoresis against human serum.

Coating

Polystyrene tubes 10.5 × 40 mm (Greiner No. 655061) were coated by incubation with a solution containing 50 mg/l antibodies against elastase in 10 mmol/l ammonium hydrogen carbonate, pH 7.8, overnight at 4 °C.

Preparation of labelled antibodies

Antibodies against α_1-proteinase inhibitor were purified from rabbit antiserum, coupled to calf intestinal alkaline phosphatase by a one step glutaraldehyde procedure, and the labelled antibodies were isolated by gel filtration as described in a separate paper (13).

Complex formation

The elastase/α_1-proteinase inhibitor complex was produced by mixing elastase with an excess of the inhibitor. Elastase (0.5 mg) and α_1-proteinase inhibitor (2.8 mg) were incubated for 30 min at 37 °C in 2 ml 30 mmol/l tris(hydroxymethyl)aminomethane/HCl buffer pH 8.0, containing 85 mmol/l NaCl. Under these conditions elastase is totally inactivated by complex formation as shown by assay of the catalytic activity using methoxysuccinyl-*L*-ala-*L*-ala-*L*-pro-*L*-val-4-nitroanilide (14), immunoelectrophoresis and polyacrylamide gel electrophoresis at pH 4.3.

The immunoassay

The assay principle is given in figure 1.

Step 1 Dilute one volume of plasma with 50 volumes of phosphate-buffered saline containing 10 g/l bovine serum albumin and 20 mmol/l EDTA.

Step 2 Incubate 500 µl sample diluent, standard or diluted sample per tube 1 h at room temperature.

Step 3 Wash three times with distilled water containing 0.5 g/l polyoxyethylene sorbitan monolaurate.

Step 4 Incubate with 500 µl reagent containing alkaline phosphatase labelled rabbit-IgG against α_1-proteinase inhibitor 1 h at room temperature.

Step 5 Wash once with distilled water containing 0.5 g/l polyoxyethylene sorbitan monolaurate.

Step 6 Incubate with 500 µl 10 mmol/l 4-nitrophenyl phosphate in 1 mol/l diethanolamine/HCl-buffer pH 9.8 containing 0.5 mmol/l $MgCl_2$.

Step 7 Stop enzyme reaction by adding 500 µl 2 mol/l NaOH.

Step 8 Read absorbance at 405 nm.

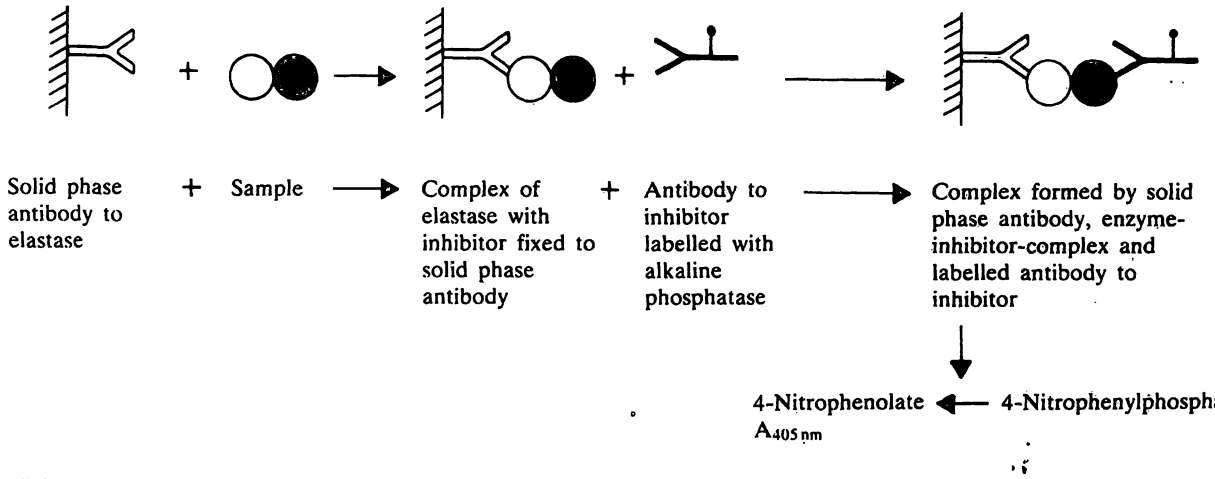

Fig. 1. Principle of solid-phase, enzyme-linked immunoassay for polymorphonuclear elastase complexed with α_1-proteinase inhibitor

Standard solutions and control plasma

Standard solutions with defined levels of complexed elastase were prepared by diluting the reaction mixture with phosphate-buffered saline containing 10 g/l bovine serum albumin. Control plasma with a known level of the complex was produced as follows: Citrated plasma from non-immunized sheep was inactivated at 56 °C for 45 min and mixed with a defined amount of elastase-α_1-proteinase inhibitor complex.

Sample collection

Citrated or EDTA plasma was used. Plasma was separated from blood within 30 min after sample collection to prevent interference by in vitro secretion by leukocytes (15, 16). Tests were performed either on fresh plasma or on aliquots stored frozen at −20 °C or −70 °C.

Results

Standard curve

The absorbance values obtained with sample diluent and the standard solutions were plotted versus concentration on a linear diagram (fig. 2). The intercept at 0 µg/l and the slope were calculated by linear regression. Absorbance readings from unknown samples were referred to the calibration curve.

The working range of the assay is 0.5 to 5.0 ng of elastase in complex with α_1-proteinase inhibitor in the assay tube. This is equal to a range of 51 to 510 µg/l in plasma when testing plasma samples diluted 1 + 50 in 500 µl final volume.

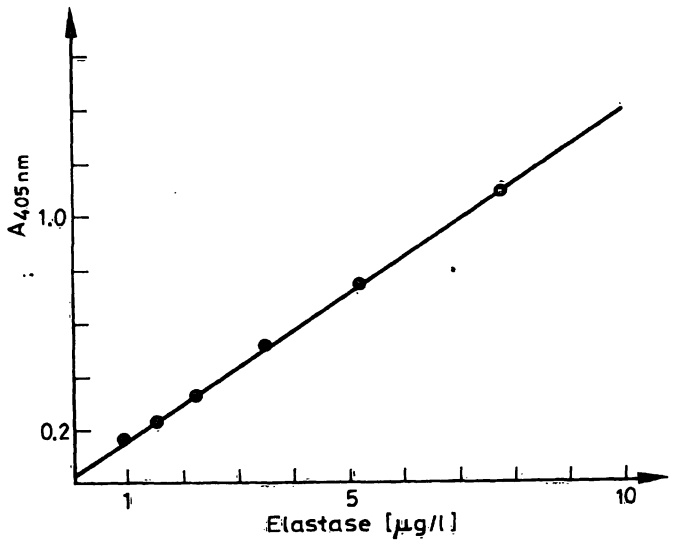

Fig. 2. Calibration curve for the elastase assay. Absorbance as a function of concentration (given as µg/l of elastase in the complex with α_1-proteinase inhibitor).

Detection limit

The lower limit of sensitivity, as defined by the mean absorbance of the blanks plus three standard deviations, was 0.25 ng per test tube. The detection limit was increased by increasing the reaction periods in Step 2 and Step 4 of the assay (see section "The immunoassay").

Precision

Precision was monitored with different plasma pools (tab. 1). Within-run imprecision (coefficient of variation) was 4 to 8%, and between-run imprecision was in the range of 3 to 8% CV.

Tab. 1. Precision of the assay.

Sample	Elastase (µg/l)	Sample volume (µl)	Series	Experiments series	CV (%)
Within-series imprecision					
Pooled plasma 1	52	20	1	15	7.7
Pooled plasma 2	206	20	1	15	3.9
Pathological plasma	389	10	1	15	4.0
Between-series imprecision					
Pooled plasma 1	47	20	15	2	8.2
Pooled plasma 2	191	20	15	2	7.4
Pathological plasma	339	10	15	2	2.9

Sample stability

Citrated plasma was stored at least 6 months at −20 °C or 14 days at +2 °C to +8 °C without appreciable loss of the antigenic reactivity of the elastase/α_1-proteinase inhibitor complex.

Linearity and parallelity

Citrated plasmas were pooled to give a plasma specimen with levels of 60 or 500 µg/l of complexed elastase. Linear results were obtained with plasma samples of 10 to 80 µl from the first pool, and with plasma samples of 1 to 8 µl from the second pool. The slopes of the plasma dilution curves were not significantly different from that of the calibration curve (fig. 3).

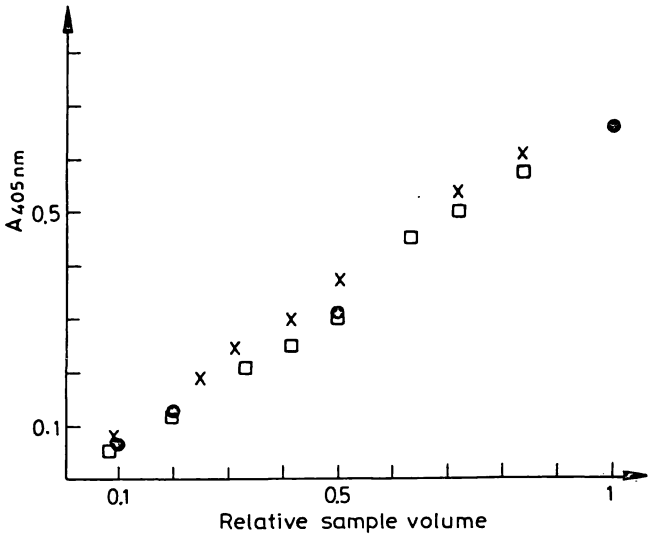

Fig. 3. Linearity and parallelity of the assay. Absorbance as a function of varying quantities of standard material or plasma.
Definition of sample volume:
●, Calibrator with 10 µg/l, 1 = 500 µl
□, Pooled human plasma with 60 µg/l, 1 = 100 µl plasma
×, Pooled human plasma with 500 µg/l, 1 = 10 µl plasma
Final volume in the test tube was adjusted to 500 µl by addition of sample diluent if necessary. From l.c. (22).

Recovery

Aliquots of the elastase/α_1-proteinase inhibitor complex as produced in vitro were added to six different citrated human plasma samples. The concentrations of the complex in the native and the spiked plasma samples were assayed. Recovery was 75 to 96 percent.

Plasma reference range

The plasma concentration of the elastase/α_1-proteinase inhibitor complex was measured in citrated plasma from 172 randomly selected, apparently healthy laboratory workers. The group consisted of 165 males and 7 females, age range 18 to 62 years. The concentration of complexed elastase was in the range 20 to 180 µg/l with a mean ± standard deviation of 67 ± 31 µg/l and a median of 64 µg/l.

Discussion

In recent years evidence has accumulated for the role of granulocyte elastase as a main pathogenetical factor in inflammatory processes. However, only a few reports have appeared on the development of quantitative laboratory assays for the determination of granulocyte elastase levels in plasma or other biological fluids. The immunoassays for granulocytic elastase reported so far are electroimmunodiffusion (4), radioimmunoassay (5, 7) and a competitive enzyme-linked immunoassay for canine granulocytic elastase (6). These determination techniques for elastase have not found wide application in the clinical chemistry laboratory.

In previous reports it was shown that granulocyte elastase in plasma is complexed mainly with α_1-proteinase inhibitor (8, 17). Elastase in this complex is devoid of enzymatic activity. A small fraction of the enzyme is also bound to α_2-macroglobulin (8). Granulocyte elastase in this complex retains some enzymatic activity towards a peptide substrate (14). Determination of this activity in plasma does not appear to be a sensitive parameter for granulocyte elastase, because the elastase/α_2-macroglobulin complex represents only a small fraction of the elastase molecules present in plasma and is eliminated very quickly from the circulation (17). We chose an alternative approach, i.e. the immunochemical quantitation of the plasma concentration of the complex of elastase with α_1-proteinase inhibitor. This technique makes use of the dual immunological specificity of the equimolar complex of an enzyme firmly bound to its inhibitor. In a first assay version microculture plates were used as carriers for the antibodies against elastase (9, 10). A similar assay which is based on the same principle was recently reported by another group (18). However, in our view, it is more convenient to use volumes of 0.5 or 1.0 ml for absorption readings on routine photometers. We therefore developed a version of the assay, which employs polystyrene tubes, and the characteristics of this assay are reported here. Both detection limits, within-series and between-series imprecision, linearity, parallelity of calibrators and plasma dilutions and recovery are within reasonable limits. Our method is sensitive, precise and practicable with the equipment of a routine clinical chemistry laboratory.

A normal range of 20 to 180 µg/l was found in a group of healthy laboratory workers. Work in progress will define the reference range on different and enlarged normal groups.

In preliminary clinical studies largely increased plasma levels were found in patients with septicaemia, polytrauma and in most patients with acute rheumatoid arthritis (3, 19, 20, 21). These data suggest a diagnostic relevance of granulocyte elastase levels in plasma.

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